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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Walter P. Carney and Sara J. McKenzie

Serial No. : ~~806,112~~ 488,180 Group Art Unit: 1802

#7 Decl

Filed : December 12, 1991 Examiner: B. Hayden

For : DETECTION AND QUANTIFICATION OF NEU RELATED PROTEINS IN THE BIOLOGICAL FLUIDS OF HUMANS

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

noted 12/22/97 MS

DECLARATION OF ARTHUR M. BRUSKIN UNDER 37 C.F.R. §1.132

I, Arthur M. Bruskin, hereby declare as follows:

1. I am Director of Cancer Research with Oncogene Science, Inc., the assignee of record of the above-identified application.

2. I am familiar with the Drebin, et al. reference, Nature 312:545-548 (1984), which was cited by the Examiner in the October 27, 1992 Office Action issued in connection with the subject application.

3. On December 3, 1990, I submitted a declaration substantially identical to this declaration in connection with U.S. Serial No. 297,188, filed January 13, 1989, of which the parent of the subject application is a continuation-in-part.

4. In December 1990, I was Group Leader for Cancer

Development Projects at Applied bioTechnology, Inc., now a subsidiary of Oncogene Science, Inc., but at that time an independent company. While I held that position, an experiment was carried out under my supervision and control to compare the specificity of the antibody referred to as 7.16.4 in the cited Drebin, et al. reference, with the specificity of the antibody to human Neu designated in the subject application. The PB3 antibody is of the same subtype as the Drebin 7.16.4 antibody and the same procedure was used with both antibodies.

5. The 7.16.4 antibody used in this experiment was obtained from Oncogene Science, Inc. which sold it as c-neu (Ab-4). A copy of the data sheet for antibody c-neu (Ab-4) which accompanied the purchased antibody is attached hereto as Exhibit 1. This data sheet shows that antibody c-neu (Ab-4) is from clone 7.16.4.

6. The following cell lines were used in this experiment:

   A. Cell lines A431, MCF-7 and SK-BR-3 were obtained from the ATCC and cultured as recommended. The ATCC catalog numbers and descriptions are attached as Exhibits 2, 3 and 4, respectively.

   B. Cell line DHFR-G8 was obtained from the laboratory of Dr. Robert Weinberg. This cell line was generated by the transfection of NIH-3T3 cells with the rat neu proto-oncogene. A copy of the data sheet for this cell line is attached hereto as Exhibit 5.

C. Cell lines A431 and MCF-7 are described in Schechter, et al., Nature 312:513-516 (1984), a copy of which is attached hereto as Exhibit 6. Table 1 on page 514 describes immunofluorescent staining of rat and human cells and indicates that antibody 7.16.4 did not stain human cell lines A431 and MCF-7.

D. Cell line DHFR-G8 expresses high levels of rat Neu protein as described by Hung, et al., Proc. Natl. Acad. Sci. 83:261-264 (1986), a copy of which is attached hereto as Exhibit 7. This reference discloses that rat Neu protein was detected by antibody 7.16.4 (See Fig. 2, lane 12).

7. The following procedure was followed in this experiment:

A. Subconfluent plates (6 cm) were incubated for 30 minutes in DMEM. The medium was replaced with 5 ml of methionine-free DMEM to which was added 250 μCi of ^{35}S-methionine. After incubation for 4 hours at 37°C the cells were lysed in 1 ml of lysis buffer (10mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 10 mM BME). After centrifugation to remove nuclei and cellular debris, the lysate was divided into two equal aliquots. 5 μg of antibody, either Ab-4 (7.16.4) or PB3 was added to the cell lysates and incubated at 4°C for 1 hour. 50 μl of protein A Sepharose was then added and the incubation was continued for an additional hour. The lysate was then diluted with 1 ml of lysis buffer and the protein A Sepharose was collected by

centrifugation. The protein A Sepharose was washed three times with 1.5 ml of RIPA buffer (10 mM Tris, pH 7.2, 500mM NaCl, 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 5 mM EDTA) and once with water. The washed protein A Sepharose was resuspended in 2X SDS sample buffer and heated at 100°C for 10 minutes. The resulting material was electrophoresed on a 7% SDS-PAGE. The resulting gel was treated with Enhance (Dupont, Cat. #NEF-981) according to the manufacturer's instructions. The dried gel was exposed to X-ray film for 4 days (or 4 hours referred to as "Short exposure"). A photocopy of the gel results is attached hereto as Exhibit 8.

8. In Exhibit 8 the lanes represent the following:

Lane 0: Markers
Lane 1: A431 lysate immunoprecipitated with Ab-4 (7.16.4).
Lane 2: A431 lysate immunoprecipitated with PB3.
Lane 3: MCF-7 lysate immunoprecipitated with Ab-4.
Lane 4: MCF-7 lysate immunoprecipitated with PB3.
Lane 5: SK-BR-3 lysate immunoprecipitated with Ab-4.
Lane 6: SK-BR-3 lysate immunoprecipitated with PB3.
Lane 7: G8 lysate immunoprecipitated with Ab-4.
Lane 8: G8 lysate immunoprecipitated with PB3.
Lane 9: SK-BR-3 lysate immunoprecipitated with PB3 (short exposure).
Lane 10: SK-BR-3 lysate immunoprecipitated with Ab-4 (short exposure).

9. The results of this experiment are displayed in the following table.

Results

Cell Line	Description	Drebin Antibody (Ab-4)	Human Neu Antibody (PB3)
A431	Human cervical carcinoma line	-	+
MCF-7	Human breast carcinoma line	-	+/*
SK-BR-3	Human breast carcinoma line	-	+
DHFR/G8	Rat neu transfected NIH3T3 cell line	+	-

+ Means binding is present
- Means antibody binding not seen
* Very small amount can be seen in original figure.

10. This experiment demonstrates that the Drebin antibody 7.16.4 does not bind human Neu. Even in the cell line SK-BR-3, which expresses high levels of human Neu, the 7.16.4 antibody does not bind human Neu. In addition, this experiment shows that the human cell lines A431 and MCF-7 express detectable levels of human Neu when assayed using the PB3 antibody. This result is in contrast to that disclosed in the Schechter, et al. reference, Exhibit 7, which indicates that antibody 7.16.4 did not stain human cell lines A431 and MCF-7. Further, as shown in Exhibit 8, lanes 7 & 8, immunoprecipitation of lysates from DHFR/G8 cells demonstrates that although rat Neu is

detected by the 7.16.4 antibody, it is not detected by the PB3 antibody.

I hereby further declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 3/26/93

Arthur M. Bruskin

Oncogene Science

MONOCLONAL ANTIBODIES TO ONCOGENE PROTEINS

c-neu (Ab-4)

BACKGROUND: Amplification of the human proto-oncogene c-*neu* (1) has been reported to occur in approximately 30% of breast carcinomas and measurement of the amplification has been proposed as a useful predictor of disease prognosis (2,3). Although there is general agreement that c-*neu* overexpression occurs in breast carcinoma the exact extent of c-*neu* involvement and its prognostic value remain controversial. Gene amplification and the resulting overexpression of proto-oncogene encoded $P185^{c\text{-}neu}$ is thought to transform cells by chronically stimulating signal transduction pathways (4,5), and in model systems overexpression of human c-*neu* induces cellular transformation (6). In the rat model system, a point mutation has been located within the transmembrane domain of the c-*neu* oncogene (7).

ORIGIN: Clone 7.16.4 (M. Green University of Pennsylvania) was derived by immunization of C_3H/HeJ mice with cells over expressing rat c-*neu* gene product, and fusion of mouse splenocytes with NS-1 myeloma cells (8).

HOW SUPPLIED: c-*neu* (Ab-4) is purified from mouse ascities fluid by non-denaturing liquid chromatography. Each vial contains 100 µg mouse IgG_{2a} in 1.0 of 0.05M sodium phosphate buffer containing 0.1% sodium azide and 0.2% gelatin. Following the protocols provided this amount of IgG should be sufficient for 100 immunoprecipitation or immunohistochemistry procedures.

Store at 4°C, do not freeze. If stored under proper conditions, the product is stable for one year from the date of shipment. For research use only, not for use in diagnostic procedures.

CHARACTERISTICS: c-*neu* (Ab-4) immunoprecipitates 185,000 molecular weight rat c-*neu* product and stains formalin-fixed paraffin-embedded tissue sections of rat cells overexpressing the c-*neu* protein (8). This antibody does not inhibit *in vitro* protein-tyrosine kinase activity.

Cat. # OP16

ATCC CRL 1547 MNNG/HOS (TE85, clone F-5)
(Chemically transformed, human osteogenic sarcoma)

†Passage Frozen: 40. Current medium for propagation: Eagle's MEM with non-essential amino acids and Earle's BSS, 90%; fetal bovine serum, 10%. Additional Information: MNNG/HOS is a chemically transformed (MNNG 0.01 mcg/ml) cell line derived from an osteogenic sarcoma from a 13 year-old, Caucasian female. It has a high saturation density, a high plating efficiency in soft agar and produces tumors in nude mice. References: Nature *256:* 751-753, 1975; Int. J. Cancer *19:* 505-510, 1977. Submitted by: J.S. Rhim, NCI, NIH. Bethesda, Md.

ATCC CRL 1548 H-4-II-E (Rat hepatoma, Reuber H35)
†Passage Frozen: 8 at ATCC. Current medium for propagation: Eagle's MEM with non-essential amino acids and Earle's BSS, 80%; fetal bovine serum, 10%; calf serum, 10%. Additional Information: This line, which has been cured of *Mycoplasma arginini* contamination, still exhibits high aryl hydrocarbon hydroxylase inducibility (In Vitro *16:* 230, 1980). It is useful as an indicator for detecting picogram amounts of biologically potent planar polychlorinated organic compounds in food extracts and other environmental samples, as well as for studies on hormone-induced enzyme synthesis. References: Nat. Cancer Inst. Monogr. *13:* 229-245, 1964; Cell Tissue Kinet. *10:* 233-243, 1977; J. Assoc. Off. Anal. Chem. *62:* 904-916, 1979; Biochem. Pharmacol. *22:* 2766-2769, 1973. Submitted by: J.A. Bradlaw, FDA, Washington, D.C.

ATCC CRL 1550 Ca Ski (Human cervical epidermoid carcinoma)
†Passage Frozen: 249. Current medium for propagation: RPMI 1640, 90%; fetal bovine serum, 10%. Additional Information: This cell line was initiated from epidermoid carcinoma of the cervix metastic to the small bowel mesentery of a 40 year-old Caucasian. The cells reportedly secrete the beta subunit of human chorionic gonadotropin (beta-hCG) and express tumor-associated antigen. The cells contain G6PD type B. Reference: Science *196:* 1456-1458, 1977. Submitted by: R.A. Pattillo, Medical College of Wisconsin, Milwaukee, Wisc.

ATCC CRL 1552 MOLT-3 (Human peripheral blood, acute lymphoblastic leukemia)
†Passage Frozen: Unknown. Current medium for propagation: RPMI 1640, 90%; fetal bovine serum, 10%. Additional Information: MOLT-3 is a suspension culture derived from the peripheral blood of a 19 year-old male with acute lymphoblastic leukemia in relapse. It is reportedly a stable T-cell leukemia; the cells bind with sheep erythrocytes to form rosettes. The terminal deoxyonucleotidyl transferase (TdT) for this cell line is high. Neither immunoglobulins nor Epstein-Barr virus were detectable. Reference: J. Nat. Cancer Inst. *49:* 891-895, 1972. Submitted by: J. Minowada, Roswell Park Memorial Institute, Buffalo, N.Y.

ATCC CRL 1554 BUD-8 (Human skin fibroblast)
†Passage Frozen: 6 (PDL unknown; 4 at ATCC). Current medium for propagation: Eagle's MEM with non-essential amino acids and Earle's BSS, 90%; fetal bovine serum, 10%. Additional Information: BUD-8 is a fibroblast-like cell line derived from the normal skin of a 56 year-old, white female. This cell line has been found to be highly sensitive to human interferon and has been proposed as a standard for its assay. References: J. Biol. Stand. *7:* 383-395, 1979. Grassberg, S.E., P. Jameson, and J.J. Sedmak. Interferon Bioassay Methods and the Development of Standard Procedures: A critique and analysis of current observations. The production and use of interferon for the treatment and prevention of human virus infections. The Tissue Culture Association, pp. 26-34, 1974. Submitted by: B.W. Uhlendorf, NIMH, Bethesda, Md.

ATCC CRL 1555 A-431 (Human epidermoid carcinoma)
†Passage Frozen: 28. Current medium for propagation: Dulbecco's modified Eagle's medium with 4.5 g/L glucose, 90%; fetal bovine serum, 10%. Additional Information: The epidermoid carcinoma cell line A-431, derived from an 85 year-old female, is one of a series of cell lines established from solid tumors by D.J. Giard, *et al.* The cell line produced rapidly growing subcutaneous tumors in antithymocyte serum-treated NIH Swiss mice and formed colonies on normal fibroblasts and in agar. Reference: J. Nat. Cancer Inst. *51:* 1417-1423, 1973. Submitted by: D.J. Giard, MIT, Cambridge, Mass. and S.A. Aaronson, NCI, NIH, Bethesda, Md.

ATCC CRL 1556 OMK(637-69) (Owl monkey kidney, *Aotus trivirgatus*)
†Passage Frozen: 14. Current medium for propagation: Eagle's MEM with non-essential amino acids and Earle's BSS, 90%; fetal bovine serum, 10%. Additional Information: The OMK cell line was established by M. Daniel and D. Jackman in November, 1970 from an apparently normal adult female Owl monkey. The line has undergone nearly 100 serial subcultivations in the deriving investigators' laboratory with no evidence of any latent agents. The cell line has been useful for propagation of many non-human primate viruses especially Herpesvirus aotus, H. saimari and H. ateles (see ATCC VR-606, 608 and 808). Submitted by: M.D. Daniel, New England Regional Primate Research Center, Harvard Medical School, Southborough, Mass.

ATCC CRL 1566 NULLI-SCC1 (Mouse teratocarcinoma)
†Passage Frozen: Unknown, 11 at ATCC. Current medium for propagation: Dulbecco's modified Eagle's medium with 4.5 g/L glucose, 90%; calf serum, 10%. Additional Information: The SCC1 clonal cell line was derived by G. Martin in August 1974 (Proc. Nat. Acad. Sci., USA *72:* 1441-1445, 1975) from mouse teratocarcinoma cells obtained from a spontaneous testicular tumor from a strain 129 mouse (J. Nat. Cancer Inst. *20:* 1257-1276, 1958). This embryonal carcinoma line does not differentiate under normal conditions *in vivo* or *in vitro*. The original tumor was a pure embryonal carcinoma with

† The passage number listed applies to material available for distribution as of December 31, 1987.

ATCC HTB 19 (continued)

Nude mouse: Forms grade II adenocarcinomas consistent with primary mammary cancer.
HLA cell line phenotype: A1; Bw16+/-.

REFERENCE SEED STOCK PREPARED AT ATCC

Number of Serial Subcultures from Tissue of Origin: 286.
Freeze Medium: Culture medium, 95%; DMSO, 5%; antibiotic-free.
Viability: 97%.
Culture Medium: Eagle's minimum essential medium with non-essential amino acids, sodium pyruvate and Earle's BSS, 90%; fetal bovine serum, 10%; antibiotic-free.
Isoenzymes: PGM_3, 1; PGM_1, 1; ES D, 1; AK1, 1-2; G6PD, B; GLO-1, 1-2.
Phenotype Frequency Product: 0.0115.
Sterility: Tests for mycoplasma, bacteria and fungi were negative.
Species: Confirmed as human by isoenzyme analysis.

ATCC HTB 20 BT-474 (Ductal carcinoma, breast, Human)

Current medium for propagation: RPMI 1640 with bovine insulin, 10 µg/ml, and glutamine, 300 mg/L, 90%; fetal bovine serum, 10%.

The BT-474 line was isolated by E. Lasfargues and W.G. Coutinho (J. Nat. Cancer Inst. *61:* 967-978, 1978) from a solid, invasive ductal carcinoma of the breast obtained from a 60 year-old female patient. The cells varied greatly in size and had large nuclei with one or more nucleoli. Microtubules, microvilli, tonofibrils, lysosomes, osmiophilic secretory granules, desmosomes, tight and gap junctions were observed in preparations examined by transmission electron microscopy. The BT-474 is reportedly tumorigenic in athymic nude mice and will form nodules in Amsterdam/IMR rats with regression in 10 days. This epithelial cell line was found to be susceptible to mouse mammary tumor virus (RIII-MuMTV) and can support its replication (In Vitro *15:* 723-729, 1979).

CHARACTERISTICS REPORTED FOR TRANSFERRED STOCK

Patient Data: Age-60; Sex-Female; Race-Caucasian.
Treatment: Surgery.
Grown as: Adherent patches of epithelial cells.
Morphology: Epithelial (compact, multi-layered colonies, rarely become confluent).
Karyology: Mode 55; range 50-112 (originator). Biomodal shift 58-59 and 100 in later passages with 3 marker chromosomes (Peterson).

REFERENCE SEED STOCK PREPARED AT ATCC

Number of Serial Subcultures from Tissue of Origin: 81.
Freeze Medium: Culture medium, 95%; DMSO, 5%.
Viability: 85-95%.
Culture Medium: RPMI 1640 medium, 90%; fetal bovine serum, 10%; bovine insulin, 10 µg/ml; glutamine, 300 mg/L.
Isoenzymes: G6PD, B; PGM_1, 1; PGM_3, 1; ES D, 1; Me-2, 0; AK1, 1; GLO-1, 1.
Phenotype Frequency Product: 0.0426.
Sterility: Tests for mycoplasma, bacteria and fungi were negative.
Species: Confirmed as human by isoenzyme analysis.

ATCC HTB 22 MCF7 (Breast adenocarcinoma, pleural effusion, Human)

Current medium for propagation: Eagle's MEM with non-essential amino acids, sodium pyruvate and Earle's BSS, 90%; fetal bovine serum, 10%.

Cells from the pleural effusion were seeded in Eagle's minimum essential medium with non-essential amino acids, 20 µg insulin per ml and 20% bovine calf serum for derivation of this line (H.D. Soule *et al.* J. Nat. Cancer Inst. *51:* 1409-1416, 1973). It has retained several characteristics of differentiated mammary epithelium including ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes.

A culture at passage 138 was donated to the ATCC by C.M. McGrath in April, 1982. He has indicated that the line may harbor B- or C-type virus information and should be handled, therefore, as a potentially biohazardous agent.

REFERENCE SEED STOCK PREPARED AT ATCC

Patient Data: Age-69; Sex-Female; Race-Caucasian; Blood Type-O+.
Treatment: Radiation and hormone therapy.
Grown as: Monolayer; transferred 1:2 weekly.
Morphology: Epithelial-like.
Karyology: Chromosome Frequency Distribution 50 Cells: 2n = 46.

Cells:	1	1	2	2	4	2	3	1	3	5	7	4	6	5	2	2
Chromosomes:	66	70	72	73	75	76	77	78	80	81	82	83	84	85	86	87

The stemline chromosome numbers ranged from hypertriploidy to hypotetraploidy, with the 2S component occurring at 1%. There were 29-34 marker chromosomes per S metaphase, of which 24-28 markers occurred in at least 30% of cells, and generally one large submetacentric (M_1) and 3 large subtelocentric (M_2, M_3, and M_4) markers were recognizable in over 80% of metaphases. No DM's were detected. Chromosome No. 20 was nullisomic and X was disomic.
Isoenzymes: PGM_3, 1; PGM_1, 1-2; ES D, 1-2; AK1, 1; GLO-1, 1-2; G6PD, B.

Isoenzymes: Me-2, 1; PGM_3, 1-2; PGM_1, 1; ES D, 1; AK1, 1; GLO-1, 2; G6PD, B.
Phenotype Frequency Product: 0.0241.
Sterility: Tests for mycoplasma, bacteria and fungi were negative.
Species: Confirmed as human by isoenzyme analysis.

ATCC HTB 30 SK-BR-3 (Adenocarcinoma, breast, malignant pleural effusion, Human)

Current medium for propagation: McCoy's 5a medium, 85%; fetal bovine serum, 15%.

This cell line was derived by G.E. Trempe and L.J. Old in 1970 from a pleural effusion. Initial cultivation was in Eagle's minimal essential medium with 10% fetal bovine serum and more recently McCoy's 5a with 10-15% fetal bovine serum. A culture at passage 6 was deposited in January 1972 by the originators.

CHARACTERISTICS REPORTED FOR TRANSFERRED STOCK

Patient Data: Age-43; Sex-Female; Race-Caucasian; Blood Type-A^+.
Treatment: Radiation, steroids, cytoxan, 5-fluorouracil.
Grown as: Monolayer; transferred 1:4 weekly.
Morphology: Epithelial-like.
Karyology: (P9) Hypertriploid to hypotetraploid (+A, +B, +C, +E, +F, +G, -D) with abnormalities including dicentrics, acrocentric fragments, rings, secondary constrictions, large metacentrics or polycentrics and large submetacentric marker (Fogh).
***In Vitro* Cytopathology:** (P16) Adenocarcinoma cells.
Nude mouse: Produces poorly differentiated adenocarcinoma consistent with breast primary grade III.
Ultrastructure: Microvilli and desmosomes, glycogen granules, large lysosomes, bundles of cytoplasmic fibrils, no virus particles (Sarkar).
HLA Cell Line Phenotype: A11; Bw22+/-, $B40^+$, $B18^+$.

REFERENCE SEED STOCK PREPARED AT ATCC

Number of Serial Subcultures from Tissue of Origin: 24.
Freeze Medium: Culture medium, 95%; DMSO, 5%; antibiotic-free.
Viability: 94%.
Culture Medium: McCoy's 5a medium (modified), 85%; fetal bovine serum, 15%.
Isoenzymes: PGM_3, 1; PGM_1, 1-2; ES D, 1; AK1, 1-2; GLO-1, 2; G6PD, B.
Phenotype Frequency Product: 0.0044.
Sterility: Tests for mycoplasma, bacteria and fungi were negative.
Species: Confirmed as human by isoenzyme analysis.

ATCC HTB 31 C-33 A (Carcinoma, cervix, Human)

Current medium for propagation: Eagle's MEM with non-essential amino acids and sodium pyruvate, 90%; fetal bovine serum, 10%.

C-33 A is one of a series of cell lines (see also ATCC CRL 1594 and 1595) derived by N. Auersperg from cervical cancer biopsies (J. Nat. Cancer Inst. *32:* 135-148, 1964). Isolated originally on Waymouth's 752/1 medium, 10% fetal bovine serum, 5-10% umbilical cord extract and 5% ascitic fluid, the line exhibited a hypodiploid karyotype initially and an epithelial morphology. Karyological instability was observed with continued passage. Marker chromosomes were described. A culture at passage 86 was provided by the originator in December of 1974.

CHARACTERISTICS REPORTED FOR TRANSFERRED STOCK

Patient Data: Age-66; Sex-Female; Race-Caucasian; Blood Type-A^+.
Grown as: Monolayer; transferred 1:10 weekly.
Morphology: Epithelial-like.
Karyology: Hypodiploid to hypotetraploid (originator).
***In Vitro* Cytopathology:** (P91) Undifferentiated carcinoma cells.
Nude mouse: Forms undifferentiated carcinoma.

REFERENCE SEED STOCK PREPARED AT ATCC

Number of Serial Subcultures from Tissue of Origin: 93.
Freeze Medium: Culture medium, 95%; DMSO, 5%; antibiotic-free.
Viability: 89%.
Culture Medium: Eagle's minimum essential medium with non-essential amino acids and sodium pyruvate, 90%; fetal bovine serum, 10%.
Isoenzymes: Me-2, 2; PGM_3, 1; PGM_1, 1; ES D, 1; AK1, 1; GLO-1, 2; G6PD, B.
Phenotype Frequency Product: 0.0039.
Sterility: Tests for mycoplasma, bacteria and fungi were negative.
Species: Confirmed as human by isoenzyme analysis.
Blood Group Antigen: Confirmed as A on cultured cells.

Cell Line Database Information

Date of Print: Friday, July 13, 1990

ID Number	Cell Name	Tissue
1573	G/DHFR-8	NIH3T3

Species	Aquired	Entry Date	Alt Name
Mouse	5/8/85	5/17/89	DHFR-G8

Freezes/Vials: 14/22
Freezes: 9026 8746 8369 7668 5334 5961 5957 5951 6485 5844 10254 5331

Genes Transferred

Non-transforming agent:
Transfection: cNu (cosmid clone of normal neu gene (G));metallothionine

Source

Mien-Chie Hung, Weinberg Lab

Reference

PNAS (86) 83:261

Culture Conditions

Media: DME
Supplements: 10% CS, 0.3 uM MTX

molecules per cell, ~100 of which therefore will be in the S_{373} form. From the quantum flux of blue light used for the stimulus (Fig. 1) and the measured S_{373} extinction in the stimulus spectral range (Fig. 2b), we calculate the S_{373} population in the average cell will absorb only about two photons in the 2-s period over which reversals were assessed. Taking the quantum efficiency for photochemical reaction to be 0.5 ± 0.2, covering the range of all known retinal pigments, only -0.7–-1.4 S_{373} molecules per cell on average would be activated photochemically by a stimulus which causes over 50% of the cells to respond. Therefore photoexcitation of a single S_{373} molecule appears to be sufficient to alter the cell's swimming behaviour.

The cell is at least an order of magnitude less sensitive to attractant than repellent light[2,3]. In our interpretation this means the amplification of the S_{373} signal by the signal transduction machinery (Fig. 4) is at least 10× greater than that of sR_{587}. The relative amplification in the two signalling pathways may depend on the specific conditions and stage of cell culture growth (unpublished observations).

The colour-sensing mechanism described here depends on the existence of two spectrally-distinct signalling forms of the same receptor. This could be a unique property of the sensory rhodopsin of halobacteria. Alternatively, the sensory rhodopsin mechanism may reflect an inherent potential of retinal/protein complexes—photoreversibility of photoconversion products of the retinal/opsin chromophore—important to the evolution of the rhodopsin family as photosensory receptors. Even retinal pigment photoproducts which are too short-lived to accumulate under physiological conditions (such as the K, L and M intermediates formed from bacteriorhodopsin[5] and the early photoproducts formed from mammalian rod rhodopsin[14]) are photoreversible. Invertebrate rhodopsins, like sensory rhodopsin and the non-retinal plant chromoprotein phytochrome, are photochromic: that is, they have photoreactions with thermal relaxation sufficiently slow to cause significant photostationary state accumulation of their photoreversible conformations[16]. The sensory rhodopsin mechanism, preserved in the archaebacterium *H. halobium*, may have provided a primitive colour-sensing capability. In the evolution of visual systems, this mechanism may have been maintained in some organisms but replaced in others by multiple receptor mechanisms with increased colour resolution.

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The *neu* oncogene: an *erb-B*-related gene encoding a 185,000-M_r tumour antigen

Alan L. Schechter*, David F. Stern*, Lalitha Vaidyanathan*, Stuart J. Decker†, Jeffrey A. Drebin‡, Mark I. Greene‡ & Robert A. Weinberg*

* Whitehead Institute for Biomedical Research, Center for Cancer Research, and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA
† The Rockefeller University, New York, New York, USA
‡ Department of Pathology, Harvard Medical School, 25 Shattuck St, Boston, Massachusetts 02115, USA

A series of rat neuro/glioblastomas all contain the same transforming gene (neu) which induces synthesis of a tumour antigen of relative molecular mass (M_r) 185,000 (p185). The neu oncogene bears homology to erb-B and the tumour antigen, p185, is serologically related to the epidermal growth factor (EGF) receptor. The two proteins, EGF receptor and p185 appear to be distinct, as they coexist in nontransformed Rat-1 cells.

GENE transfer (transfection) has revealed the presence of oncogenes in the genomes of a variety of spontaneous and chemically induced tumours. Many of these oncogenes are allelic versions of one of the three genes belonging to the *ras* gene family. In several cases, the functional differences between these oncogenes and their cognate proto-oncogenes derive from single nucleotide changes, mutations which apparently have occurred in the target tissue as one of the steps of carcinogenesis (reviewed in refs 1, 2).

We have been investigating a family of transforming genes in the genomes of several rat neuro/glioblastoma cell lines derived from tumours induced by ethylnitrosourea (ENU)[3]. Injection of pregnant BDIX rats with this carcinogen during day 15 of gestation results in the appearance of neuro/glioblastomas in the offspring. DNA prepared from four neuro/glioblastoma cell lines transforms NIH 3T3 cells when introduced by transfection[4], but Southern blot analysis of the genomes of the transformed cells gave no indication of the presence of acquired *ras* oncogenes (A.L.S. and L.V., unpublished results), suggesting that a *ras* gene was not involved in the transforming activity.

The four independently activated neuro/glioblastoma oncogenes exhibited a common pattern of sensitivity or resistance to inactivation by restriction enzyme cleavage (C. Shih and R.A.W., unpublished data), suggesting that all are allelic versions of a common antecedent gene. We have applied the term '*neu*' to this group of apparently related genes.

These neuro/glioblastoma oncogenes are associated with the expression of a common tumour antigen[5]. NIH 3T3 cells

transformed by *neu* oncogenes were used to seed tumours in young mice, the sera from which precipitated a phosphoprotein of relative molecular mass (M_r) 185,000 (185K) from lysates of all NIH 3T3 lines that carry the neuro/glioblastoma oncogenes. This protein, designated p185, was detected neither in untransformed NIH 3T3 cells nor in NIH cells transformed by various *ras* oncogenes. Furthermore, when *ras*-transformed NIH 3T3 cells were used to seed tumours in mice, no serum reactivity to p185 was induced. Thus, synthesis of p185 is strongly associated with the presence of the neuro/glioblastoma oncogenes[5].

Results from several laboratories have shown that the cellular homologues of certain oncogenes first identified in retroviral genomes are also important in the genesis of spontaneous and chemically induced tumours (reviewed in refs 1, 2). One of these retrovirus-associated oncogenes is *erb-B*, which was recently shown to be homologous with the cellular gene encoding the epidermal growth factor (EGF) receptor[3]. The EGF receptor and p185 have similar molecular weights, and they are both phosphorylated cell-surface glycoproteins (refs 7–11 and A.L.S., D.F.S. and R. Negrin, unpublished data). Thus, we suspected a possible relationship between *erb-B* and the *neu* oncogenes. We report here on the homology of the *neu* and *erb-B* genes and on the relationship of p185 to an *erb-B* gene product, the EGF receptor.

Homology with *erb-B*

Possible homology between *erb-B* and *neu* was tested by Southern blot hybridization. DNA was prepared from a panel of NIH 3T3 cells, each of which had been transformed by DNA from one of the rat neuro/glioblastoma cell lines. The DNAs were digested with endonuclease *Eco*RI before electrophoresis. Figure 1*c–p* shows that NIH 3T3 cells that have been transformed by the neuro/glioblastoma DNA carry an *Eco*RI fragment that reacts with an *erb-B*-specific sequence probe. The variability in size of the new *Eco*RI fragments probably results from rearrangements in sequences flanking the transforming gene which occur during transfection. Such variability has been observed previously in cells transfected with the EJ bladder carcinoma oncogene[12]. An *erb-B*-homologous fragment of similar size is present in much smaller amounts in untransfected NIH 3T3 cells (Fig. 1*q*).

To distinguish between the rat and mouse *erb-B* homologues and to ensure that the large *Eco*RI segments shown in Fig. 1*c–p* are of donor rat origin, we treated the DNA of the transfected cells with endonucleases *Eco*RI and *Bam*HI before Southern blot analysis. This analysis confirmed that the transfected cells carry an acquired 4.4-kilobase(kb) *BAM*/RI DNA segment which has no counterpart in the genome of the recipient mouse cells (Fig. 2). Thus, 14 independently transfected cell lines shown to express high levels of p185 have acquired a DNA segment exhibiting some homology with the *erb-B* probe. Because these

Fig. 1 Southern blot analysis of *erb-B*-related sequences in NIH 3T3 cells transformed with rat neuro/glioblastoma DNAs: *Eco*RI digestion. The DNAs analysed were obtained from the following: *a*, BDIX rat liver; *b*, B104; *c*, *d*, primary transfectants of B104 (B104-1 and B104-3); *e*, *f*, secondary transfectants of B104 (B104-1-1 and B104-1-2); *g*, *h*, primary transfectants of B103 (B103-5 and B103-11); *i*, *j*, secondary transfectants of B103 (B103-1-1 and B103-1-2); *k*, B50-1, a primary transfectant of B50; *l*, *m*, secondary transfectants of B50 (B50 6a-2 and B50 6a-3); *n*, *o*, primary transfectants of B82 (B82-1 and B82-3); *p*, B82-3-2, a secondary transfectant of B82; *q*, NIH 3T3. M_r standards comprising λ phage *Hin*dIII restriction fragments are shown on the left.
Methods: High-molecular weight DNA (10 μg) was digested with endonuclease *Eco*RI, fractionated by electrophoresis in 0.9% agarose gels, and transferred to nitrocellulose paper. The filters were hybridized at low stringency (30% formamide, 5×SSCPE, 42 °C) for 36 h with an *erb* specific probe (a *Sac*I–*Pvu*II 1.8-kb subclone of avian erythroblastosis virus[22]) nick-translated with ^{32}P-dCTP. The filters were then washed three times at 20 °C for 10 min and for 5 h at 50 °C in 2×SSCPE. The filters were exposed to Kodak X-Omat AR for 3 days at −70 °C.

Table 1 Immunofluorescent staining of rat and human cell lines by antibody 7.16.4

Cell lines	Description		Immunofluorescence
B104	*neu*$^+$ rat neuroblastoma	*neu* detected by transfection	+
B82	*neu*$^+$ rat neuroblastoma	*neu* detected by transfection	+
B50	*neu*$^+$ rat neuroblastoma	*neu* detected by transfection	+++
B15	*neu*$^-$ rat neuroblastoma	*neu* not detected by transfection	++
B65	*neu*$^-$ rat neuroblastoma	*neu* not detected by transfection	++++
B104-1-1	NIH 3T3 transfectant of *neu*$^+$ DNA		-
NIH 3T3	Mouse fibroblast cell line		++++
Rat1 B104)-2	Rat-1 transfectant of *neu*$^+$ DNA		+++
Rat1 B104)-3	Rat-1 transfectant of *neu*$^+$ DNA		+++
Rat1 B104)-4	Rat-1 transfectant of *neu*$^+$ DNA		+
Rat-1	Rat fibroblast cell line		++
WTCL4	Rous sarcoma virus-induced rat neuroblastoma		-
A431	Human cervical carcinoma line		-
PC3	Human prostate carcinoma line		-
MCF7	Human breast carcinoma line		-
EJ	Human bladder carcinoma line		-
G361	Human colon carcinoma line		-
H1135	Human melanoma line		-

Cell lines were prepared for FACS analysis on a Coulter Epics V Cell Sorter as described in ref. 11. Immunofluorescence was quantified by subtracting the median fluorescence channel of populations stained with FITC-conjugated rabbit anti-mouse immunoglobulin alone (negative control) from the median fluorescence channel of populations stained with monoclonal antibody 7.16.4 followed by FITC-conjugated rabbit anti-mouse (positive staining). The resulting net fluorescence channel was expressed according to the following scale: -, less than two channels; +, 5–10 channels; ++, 11–15 channels; +++, 16–20 channels; ++++, >20 channels.

cell lines derive from several independent transfections of four different neuro/glioblastoma tumour DNAs, we concluded, as we had speculated, that a common oncogene is present in the genomes of the four donor neuro/glioblastomas.

The *erb-B* oncogene probe reacts with at least two fragments produced by *Eco*RI cleavage of rat DNA (Fig. 1*a*, *b*) which are respectively 7 and 34 kb long (M-C. Hung and A.L.S., unpublished results). The 34-kb fragment is of identical size to the rat DNA segment found in transfected NIH 3T3 cells and must represent the normal counterpart to the DNA segment bearing the *neu* oncogenes. Because *Eco*RI does not inactivate the *neu* oncogene (C. Shih and R.A.W., unpublished results), we conclude that this oncogene lies entirely within the 34-kb *Eco*RI fragment. The 7-kb *erb-B*-related DNA segment may represent a distinct but related gene. The Southern blot analysis does not indicate which of these *erb-B*-homologous segments encodes the EGF receptor, so that we could not confirm whether the *neu* gene is identical with, or only related to, the gene encoding the EGF receptor.

p185 and EGF receptor are related

The homology between *erb-B* and *neu* suggested that the EGF receptor, a known *erb-B* gene product, might be related to p185, the protein induced in cells transfected with *neu*. Two different antibody preparations were used to examine this possibility. Monoclonal antibody 7.16.4 was derived from mice immunized with B104-1-1 cells (a cell line derived by two serial passages of the *neu* oncogene through NIH 3T3 cells; ref. 4). The reactivities of the 7.16.4 antibody are indicated by the immunofluoresence data shown in Table 1. The antibody recognizes a cell-surface determinant displayed by rat neuro/glioblastomas, by a variety of normal and transformed rat cell lines and by NIH 3T3 cells transformed by the rat oncogene; it does not react with normal NIH 3T3 cells or with a variety of human tumour cell types, including the A431 cells known[13,14] to display large amounts of EGF receptor. The 7.16.4 antibody appears to react with a rat-specific determinant of p185. Further characterization of this antibody is reported elsewhere[11]. The second source of antibody was polyclonal anti-EGF receptor serum raised against EGF receptor purified from human A431 cells; this serum reacts with human and rodent EGF receptor[15].

We used these sera to elucidate the relationship between the EGF receptor and p185. Lysates of B104-1-1 cells, metabolically labelled with ^{35}S-cysteine, were incubated with normal mouse serum, serum 7.16.4 or anti-EGF receptor serum; p185 was precipitated by the 7.16.4 serum (Fig. 3*h*), but not by the control serum (Fig. 3*g*). Incubation with anti-EGF receptor serum (Fig. 3*i*) yielded a protein that co-migrated with p185. This suggested that the p185 induced by the rat *neu* oncogene shares antigenic determinants with the human EGF receptor.

To confirm that the two immune sera recognize the same protein in B104-1-1 cells, labelled lysates cleared by incubation with one antibody were subsequently reacted with the other. For example, the supernatant remaining after incubation with antibody 7.16.4 was then reacted with the anti-EGF receptor serum, which did not precipitate a 185K protein from the lysate (Fig. 3*l*). However, anti-EGF receptor did precipitate the 185K protein from a lysate cleared with normal serum (Fig. 3*k*). Similarly, p185 could be precipitated by 7.16.4 serum from a lysate that had been cleared with normal serum (Fig. 3*m*), but not from a lysate that had been cleared with anti-EGF receptor serum (Fig. 3*n*). (The decreased intensity of specific and nonspecific bands in lanes *k* to *n* of Fig. 3 compared with lanes *g* to *j* indicates that nonspecific losses occurred during the second round of immunoprecipitation.) These results confirm that an anti-EGF receptor serum reacts with p185. Thus, we conclude that p185 and EGF receptor have antigenic determinants in common.

Although p185 and EGF receptor are serologically related, they have different electrophoretic mobilities. The rat EGF receptor has an apparent M_r of ~170,000, while p185 is some-

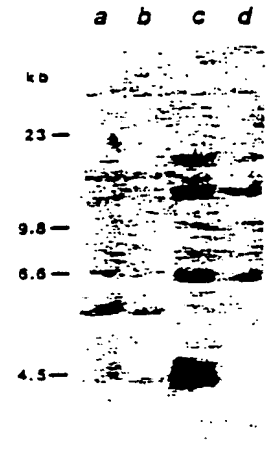

Fig. 2 Southern blot analysis of *erb-B*-related sequences: *Eco*RI plus *Bam*HI digestion. The DNAs analysed were: *a*, BDIX rat liver; *b*, B104; *c*, B104-1; *d*, NIH 3T3. High-molecular weight DNA (10 μg) was digested with restriction endonucleases *Eco*RI and *Bam*HI and analysed as described in Fig. 1 legend.

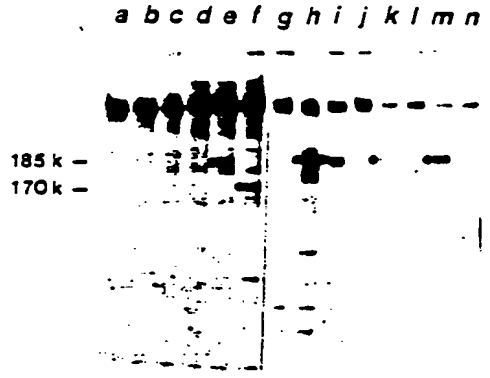

Fig. 3 Immunoprecipitation of p185 and EGF receptor. Lysates of ^{35}S-cysteine-labelled cells were incubated with antibody and subsequently precipitated. Lane *a*, NIH 3T3 cells incubated with normal mouse serum (NMS); *b*, NIH 3T3 cells incubated with antibody 7.16.4; *c*, NIH 3T3 cells incubated with anti-EGF receptor antiserum; *d*, Rat-1 cells incubated with NMS; *e*, Rat-1 incubated with antibody 7.16.4; *f*, Rat-1 incubated with anti-EGF receptor serum. *g*–*n*, B104-1-1 incubated with the following: *g*, NMS; *h*, antibody 7.16.4; *i*, anti-EGF receptor serum; *j*, NMS; *k*, supernatant from *g*, incubated with anti-EGF receptor; *l*, supernatant from *h*, incubated with anti-EGF receptor; *m*, supernatant from *j*, incubated with antibody 7.16.4; *n*, supernatant from *i*, incubated with antibody 7.16.4.

Methods: 3×10^6 cells (B104-1-1) or 3×10^5 cells (NIH 3T3 and Rat-1) were seeded in 60-mm culture dishes and labelled for 18 h in 0.8 ml of Hank's minimal essential medium containing 0.1 the usual amount of cysteine, 2% dialysed fetal calf serum, and 250 μCi ^{35}S-cysteine (977 Ci mmol^{-1}; NEN) per ml. Cells were lysed in phosphate-buffered RIPA buffer containing 1 mM ATP, 2 mM EDTA and 20 mM sodium fluoride and immunoprecipitates prepared and washed as described elsewhere[13]. Portions of the lysates were incubated with NMS (1 μl), antibody 7.16.4 (concentrated, 1 μl) or 3 μl anti-EGF receptor antiserum for 60 min, then with 1 μl of sheep anti-mouse immunoglobulin (Cappel). Incubation was continued for 30 min, after which immunoprecipitates were collected using fixed *Staphylococcus aureus*. Precipitates were prepared from 300,000 cells (lanes *a*–*f*), 60,000 cells (*g*, *h*, *k* and *l*) or 30,000 cells (*i*, *j*, *m* and *n*). (Samples in *g*–*n* were all derived from the same original lysate.) Precipitates in lanes *k* and *l* were prepared from supernatants remaining after precipitation of the lysates shown in lanes *g* and *h*. Precipitates in lanes *m* and *n* were prepared from lysates first precipitated to yield the samples shown in lanes *j* and *i*, respectively. The samples were analysed by SDS-polyacrylamide gel electrophoresis in a 7.5% acrylamide–0.17% bisacrylamide gel. The gel was fluorographed[24] and exposed to preflashed film for 7 days at −70 °C.

what larger: this difference could arise from two sources. It is possible that the mutation(s) leading to activation of the *neu* oncogene grossly affect the structure of the encoded protein so that a gene normally encoding an EGF receptor-sized protein might, after oncogenic activation, encode a protein the size of p185. Alternatively, the *neu* oncogenes might derive from a distinct proto-oncogene that normally encodes a 185K protein.

To distinguish between these possibilities, we examined two nontransformed fibroblast cell lines, hoping to identify possible normal protein homologues of p185. We have not yet been able to identify large proteins specifically precipitated from NIH 3T3 cells by either antibody 7.16.4 or anti-EGF receptor serum (Fig. 3*a–c*). However, these antibodies do recognize proteins present at low levels in untransformed Rat-1 cells. The anti-EGF receptor preferentially precipitates a protein of 170K (Fig. 3*f*) which has a slightly greater mobility than the EGF receptor precipitated from A431 cells (D.F.S., unpublished results) and is probably the rat EGF receptor. In contrast, antibody 7.16.4 precipitates a protein (Fig. 3*e*) that migrates slightly more rapidly than p185 (lanes *h*, *i*), but this antibody does not precipitate the putative EGF receptor. These data indicate that the two sera recognize distinct proteins in Rat-1 cells, and support the notion that p185, induced by the *neu* oncogene, stems from a protein of similar size present in normal cells. The normal protein may be distinct from the EGF receptor.

Implications

The *neu* gene was found to be activated in cell lines derived from four of six independent rat neuro/glioblastomas, the DNAs of which were tested by transfection[4]. Thus, *neu* activation is a frequent occurrence when the ENU pulse carcinogenesis protocol is used to induce neuroectodermal tumours in rats. Induction of tumours in rodents by defined chemical or radiological regimes has, in other instances, resulted in reproducible activation of particular oncogenes[16,17], but, hitherto, only *ras* gene activation has been reported.

The activation of the *neu* oncogenes in ENU-treated rats is of special interest because this experimental model of carcinogenesis has been characterized in great detail. These neuro/glioblastomas are induced by exposure to ENU during a well-defined window of time in late fetal and early postnatal development. Studies by Rajewsky and co-workers indicate that proliferation of target blast cells and the absence of a DNA repair system in embryonic rat brain cells strongly influence the kinetics of tumour induction[18]. Moreover, these investigators have developed an *in vitro* culture system in which brain cells are explanted from fetal rats following transplacental mutagenesis with ENU. These cultures undergo a series of changes and ultimately yield tumorigenic clones with the same lag period as that required for induction of tumours *in vivo*[19]. By using this culture system, it may be possible to determine whether *neu* activation is an early or late event in tumorigenesis, and whether early identification of premalignant cells is possible using p185-specific antibodies.

We have found that there is nucleic acid sequence homology between *neu* and *erb-B* oncogenes, and that the EGF receptor, encoded by an *erb-B*-related gene, is antigenically related to the *neu*-associated tumour antigen, p185. These results strongly suggest that p185 is a product of the *neu* oncogene.

The *erb-B* probe recognizes at least two DNA fragments in the rat genome, one of which encompasses a biologically active *neu* oncogene. This raises the possibility that there may be more than one functional *erb-B*-related gene in the rat genome, and that the gene which specifies EGF receptor is partially or completely separate from the *neu* proto-oncogene.

The data of Fig. 2 indicate that the anti-EGF receptor and 7.16.4 antibodies recognize distinct proteins that coexist in nontransformed Rat-1 cells. This suggests that the differences between the EGF receptor and a p185-like protein can be observed in cells that have not undergone oncogenic transformation. We consider it possible that the two proteins, while related to one another in structure, are made on distinct templates within the same cell and derive, at least in part, from distinct sequences in the DNA. Further structural characterization of the two proteins is under way.

Whether *neu* is an allele of the gene that encodes EGF receptor or a second related gene, the similarities between p185 and EGF receptor suggest that the normal *neu* gene product is a growth factor receptor. We speculate that the *neu* transforming protein functions by delivering a mitogenic signal to the interior of the cell, even in the absence of appropriate stimulation by the cognate growth factor—this would be analogous to the finding that the *v-erb-B* gene product lacks the EGF-binding domain of the receptor and must therefore be insensitive to the presence of EGF[7].

The role of growth factor receptors in spontaneous tumorigenesis was previously inferred from the findings that a human vulval carcinoma cell line (A431)[13,14], human squamous cell carcinomas[20] and human glial tumours[21] have large numbers of surface EGF receptors. Taken together with our findings, these results indicate that activation of growth factor receptors may be a frequent occurrence in tumorigenesis. Unlike the *ras* gene product, polypeptide growth factor receptors possess extracellular domains. It is possible that altered growth factor receptors bear novel tumour-specific antigens that can be identified by monospecific antisera. In contrast to most tumour-associated surface antigens, which are probably incidental to the transformation of a cell, the expression of p185 and analogous proteins seems to be intrinsic to the establishment and maintenance of the transformed state. This may make it possible specifically to alter the growth properties of these cells with immunological reagents that recognize determinants expressed on their surface.

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Biochemistry

Molecular cloning of the *neu* gene: Absence of gross structural alteration in oncogenic alleles

(growth factor receptor/neuroblastoma/gene amplification)

MIEN-CHIE HUNG, ALAN L. SCHECHTER, PIERRE-YVES M. CHEVRAY, DAVID F. STERN, AND ROBERT A. WEINBERG

Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142; and Biology Department, Massachusetts Institute of Technology, Cambridge, MA 02139

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ABSTRACT **The *neu* gene is distantly related to the *erbB* gene and encodes a cell surface protein that appears to function as a growth factor receptor. To study the mechanisms that caused the conversion of the normal *neu* gene to an oncogenic allele, we have isolated molecular clones of the *neu* oncogene as well as a clone of the corresponding protooncogene. The transforming *neu* oncogene and the proto-*neu* gene clones exhibit identical restriction enzyme patterns. Amplification of the proto-*neu* gene in NIH 3T3 cells by means of cotransfection with a dihydrofolate reductase gene resulted in methotrexate-resistant colonies that produce high levels of normal *neu*-encoded p185 protein. In contrast to cells carrying low levels of the oncogene-encoded protein, these cells appeared normal. The results suggest that the lesion that led to activation of the *neu* gene is a minor change in DNA sequence and is apparently located in the protein-encoding region of the gene.**

Rat neuro/glioblastomas induced by transplacental injection of ethylnitrosourea frequently carry an oncogene that is detectable by transfection into mouse NIH 3T3 cells (1, 2). We have designated this oncogene *neu* (3). The transfected NIH 3T3 cells display a novel 185,000-dalton tumor antigen (p185) that is not detected when these recipient cells are transformed by other oncogenes (4). The *neu* oncogene was shown to have some sequence homology with c-*erbB*, the gene encoding the structure of the epidermal growth factor receptor (EGF-r) (5). In addition, the encoded product of *neu*, the p185 antigen, is serologically related to the EGF-r (3). The *neu* gene in the normal and oncogenic forms appears to be distinct from the gene that encodes the EGF-r (6).

The genes encoding two growth factor receptors, the EGF-r and the receptor for the mononuclear phagocyte growth factor CSF-1, have been found to be activated into oncogenes (7, 8). In each case, these activations have involved major changes in the structure of these genes.

As an initial step toward studying the activating mechanism(s) of *neu*, we have used molecular cloning to isolate the oncogenic alleles of the *neu* gene from two rat neuro/glioblastomas cell lines and the normal allele from rat liver DNA. The present results indicate that oncogenic activation of the *neu* gene in these rat tumors occurred by means of quite different processes that creates minimal changes in protein structure.

MATERIALS AND METHODS

Construction and Screening of Cosmid Library. Cosmid libraries were constructed essentially according to the protocol of Ish-Horowitz and Burke (9). Genomic DNA was isolated from the cell lines, digested with *Eco*RI to completion, and loaded onto a 13-ml 1.25–5.0 M NaCl (10 mM Tris·HCl, pH 8.0/1.0 mM EDTA) exponential gradient. Centrifugation was for 4 hr at 40,000 rpm at 25°C in an SW 40.1 Beckman rotor. Twelve fractions were collected, aliquots were taken, and the size of the DNA was analyzed by gel electrophoresis. Three fractions containing DNA in the size range of 30–40 kilobases (kb) were combined.

The 30- to 40-kb *Eco*RI segments were ligated to the arms of the cosmid vector pSAE (10), as generated by partial *Eco*RI digestion. The DNA was packaged by using the extract and protocol from Promega-Biotec (Madison, WI). Transduction was carried out as described by Grosveld *et al.* (10) with *Escherichia coli* 1046 as host.

The library was screened according to published techniques (11), using as probe a gel-purified *Sac* I/*Pvu* II fragment of avian erythroblastosis virus (12). This probe is *erbB* specific and reacts with DNA containing the *neu* oncogene (3). Hybridization was carried out at low stringency (30% formamide/0.75 M NaCl/75 mM sodium citrate, pH 7.0, 42°C) for 36 hr. The filters then were washed three times at 20°C for 10 min and at 50°C for 4 hr with 0.3 M NaCl/30 mM sodium citrate/0.1% $NaDodSO_4$.

Southern Analysis. Southern blot analysis was performed essentially according to published techniques (11). The nitrocellulose filters were hybridized at high stringency (50% formamide/0.75 M NaCl/75 mM sodium citrate, 42°C) for 36 hr and then were washed twice at 20°C for 5 min in 0.3 M NaCl/30 mM sodium citrate and at 65°C for 1 hr in 15 mM NaCl/1.5 mM sodium citrate/0.1% $NaDodSO_4$.

Immunoprecipitation. Immunoprecipitation of p185 was carried out by using lysates of cells that were metabolically labeled with [^{35}S]cysteine as described in detail (3).

Transfection of Cloned DNA. DNA transfection into mouse NIH 3T3 cells was carried out by the calcium phosphate precipitation technique of Graham and van der Eb (13) as modified by Anderson *et al.* (14). After 2 weeks, foci of morphologically transformed cells were scored and analyzed. Cotransfection with pSV2-DHFR* plasmid (kindly provided by Heidi Stuhlmann) was performed at a molar ratio of 1:10 with the cNeu-p clone carrying the normal allele of *neu* according to published procedures (15). The pSV2-DHFR* plasmid contains an altered mouse dihydrofolate reductase (DHFR) cDNA (15) that displays abnormally low affinity for methotrexate (MTX). NIH 3T3 cells (8×10^5) in a 10-cm plate were exposed to 10 μg of the cNeu-p clone and 0.1 μg of the pSV2-DHFR* clone. After 24 hr the cells were split into 10 10-cm plates and grown in the presence of 0.6 μM MTX in Dulbecco's modified Eagle's medium containing 10% dialyzed calf serum. The medium was changed every 3–4 days.

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Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate; kb, kilobase(s); EGF-r, epidermal growth factor receptor.

The MTX-resistant colonies were subcloned with glass cylinder cloning rings after 12–14 days.

RESULTS

We wished to obtain a molecular clone of the *neu* oncogene that demonstrated transforming activity. Our previous work showed that the biological activity of the *neu* oncogene is not inactivated by endonuclease *Eco*RI (1). This suggested that the entirety of the gene lay within a single *Eco*RI segment whose size we estimated was >23 kb (3). Consequently, we attempted to isolate by molecular cloning this *Eco*RI segment with the hope that it would contain a biologically intact *neu* oncogene.

In devising a cloning strategy, an accurate size determination of this *Eco*RI segment was needed. To obtain this, we studied DNAs from B103-1 and B104-1 cell lines that had been transfected with B103 and B104 tumor DNAs, respectively. These DNAs were digested with *Eco*RI and size-fractionated by electrophoresis through low-composition (0.4%) agarose gels. Analysis by Southern blotting using an *erbB* probe (12) revealed that the large *neu*-containing *Eco*RI segments were about 33 kb in size (data not shown). Because λ phage vectors are unable to carry DNA segments of this size, we therefore turned to a cosmid vector capable of packaging DNA within the 30- to 40-kb size range. In particular, we chose to employ the cosmid vector pSAE (10) since arms generated by partial *Eco*RI cleavage of its DNA should be able to carry insert DNA in the range of 30–40 kb.

Libraries were constructed from the genomes of the two NIH 3T3 transfectants deriving from transfer of the B103 and the B104 neuroblastoma DNAs since these transfections resulted in the insertion of multiple copies of the *neu* oncogene in the recipient cell genomes (3). We further attempted to package only *Eco*RI-digested DNA of 30–40 kb in size that had been enriched by gradient centrifugation. The resulting cosmid libraries were screened with an *erbB* probe that is crossreactive with *neu* (3). Each of the positive clones, designated cNeu-103 and cNeu-104, contains a 33-kb *Eco*RI insert.

We wished to determine whether these clones were capa ble of oncogenic transformation of NIH 3T3 cells. To tha end, we transfected 1 μg of the cloned DNA by the calciun phosphate precipitation technique (13) and after 2 week scored foci of transformed cells. Approximately 300 foc appeared in the monolayer cultures of NIH 3T3 cells that ha been transfected with the cNeu-103 or the cNeu-104 clone These foci largely contained cells that were extremel refractile. Fig. 1 shows representative cell lines derived fron such foci.

Further work confirmed that these foci of transformed cell contain the intact transfected *neu* DNA and the encode tumor antigen p185. Fig. 2 shows a Southern blot analysis c the DNAs of these cell lines and immunoprecipitation o metabolically labeled cell lysates. As can be seen, th putative transfectants all carry the 33-kb *Eco*RI DNA seg ments associated with the *neu* oncogene and express th oncogene-encoded p185 protein.

To study the activating mechanism of *neu*, we isolated molecular clone of the normal allele of *neu* by screening cosmid library constructed from normal BDIX rat liver DNA The resulting clone, designated cNeu-p, also carries a 33-k *Eco*RI insert that exhibits the same restriction pattern as th cNeu-103 (Fig. 3). In fact, analysis of 50 restriction enzym sites revealed no differences between the normal an oncogenic clones of the gene (not shown).

The oncogenic transforming ability of the normal clone c *neu* was tested by transfection into NIH 3T3 cells. No fo could be detected. Cotransfection of this normal clone wit the neomycin-resistance gene resulted in colonies that pr duce a p185 antigen that migrates similarly to the antige found in oncogene-transformed cells (see below). These cel were not morphologically altered. Thus, NIH 3T3 cells th have obtained the normal rat allele by means of transfectio express a normal version of the rat p185 that apparently lack the ability to induce morphological transformation.

These data suggested that a subtle change in the structur of the *neu* gene resulted in its conversion to an activ oncogene. Other growth factor receptor genes have bee converted to active oncogenes following gene amplificatio or mutations that cause substantial rearrangement in protei

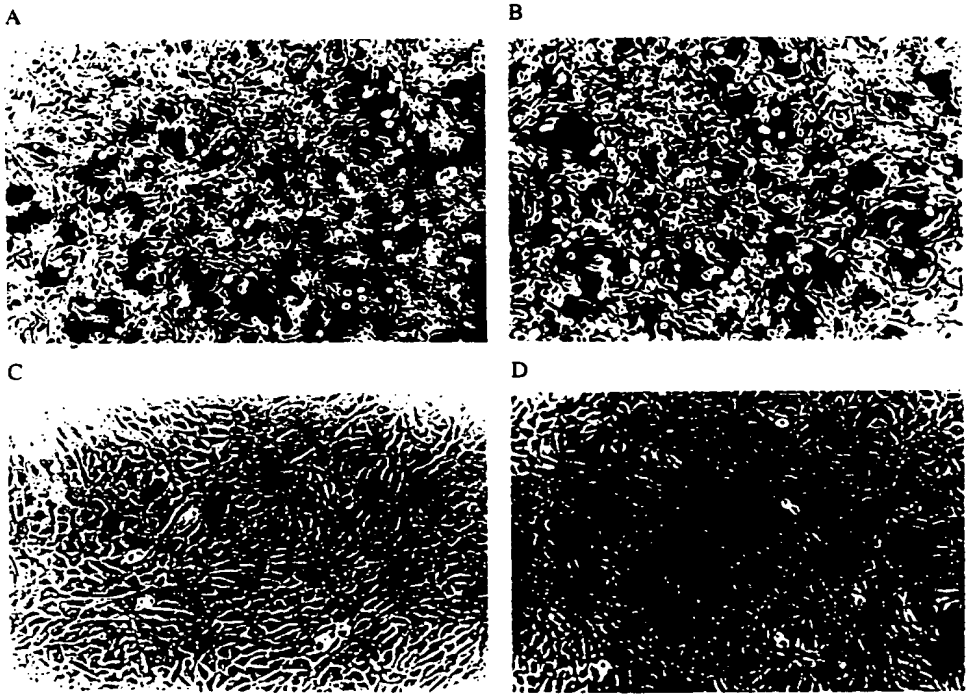

FIG. 1. Morphology of *neu* NIH 3T3. (*A*) cNeu-103-1, a transfectant containing the cNeu-103 clone. (*B*) cNeu-104-1, a transfectant containing the cNeu-104 clone. (*C*) DHFR/G-8, a transfectant containing the cNeu-p clone and pSV2-DHFR* plasmid. (*D*) Untransfected recipient NIH 3T3 cells. (×50.)

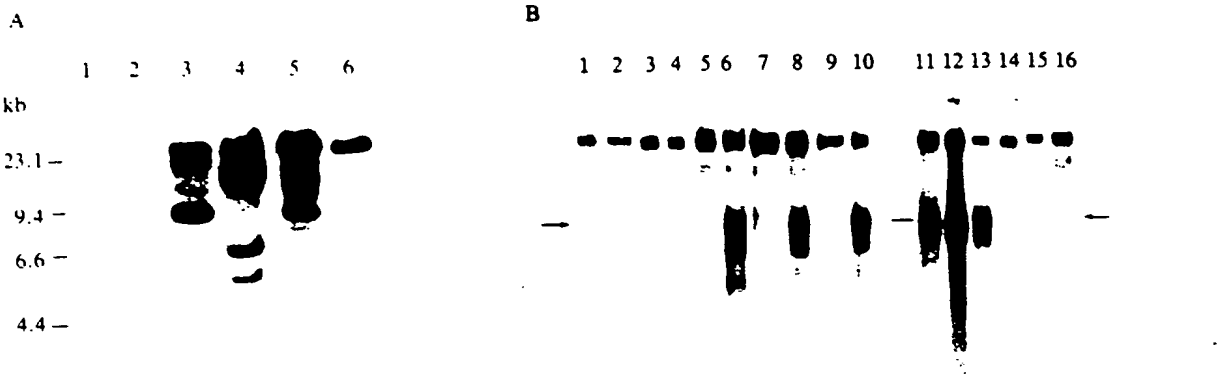

FIG. 2. (*A*) Southern analysis of *neu* NIH 3T3 transfectants. DNA was isolated from indicated sources, digested with *Eco*RI, and analyzed by Southern blotting probing with a 4.0-kb *Bam*HI segment of cNeu-103 that has been shown to be *erbB* homologous (3). Ten micrograms of DNA was loaded on each lane. Lane 1, BDIX rat liver; lane 2, NIH 3T3 cell; lane 3, cNeu-103-1, a transfectant containing the cNeu-103 clone; lane 4, cNeu-104-1, a transfectant containing the cNeu-104 clone; lane 5, DHFR/G-8, a cell line derived from cotransfection of cNeu-p and pSV2-DHFR*; lane 6, B104-1. (*B*) Immunoprecipitation of p185. Lysates of [^{35}S]cysteine-labeled cells were incubated with either preimmune (lanes 1, 3, 5, 7, 9, and 14–16) or anti-p185 monoclonal antibody 7.16.4 (16) (lanes 2, 4, 6, 8, and 10–13) and subsequently precipitated and analyzed by NaDodSO$_4$ gel electrophoresis. Lanes 1 and 2, Rat-1 cells; lanes 3 and 4, NIH 3T3 cells; lanes 5 and 6, cNeu-103-1; lanes 7 and 8, cNeu-104-1; lanes 9, 10, 13, and 16, B104-1; lanes 11 and 14, DHFR/G-6; lanes 12 and 15, DHFR/G-8; DHFR/G-6 and DHFR/G-8 are two independent cell lines containing cNeu-p and pSV2-DHFR*. The arrows show the position of p185.

...ructure (7, 8, 17, 18). We wished to see whether a dereg-...ation of *neu* expression, such as that which might arise following gene amplification, might explain the transforming powers of this gene. We therefore selected cells containing a high copy number of the normal *neu* clone by cotransfection of this clone with the pSV2-DHFR*, a clone of the DHFR gene.

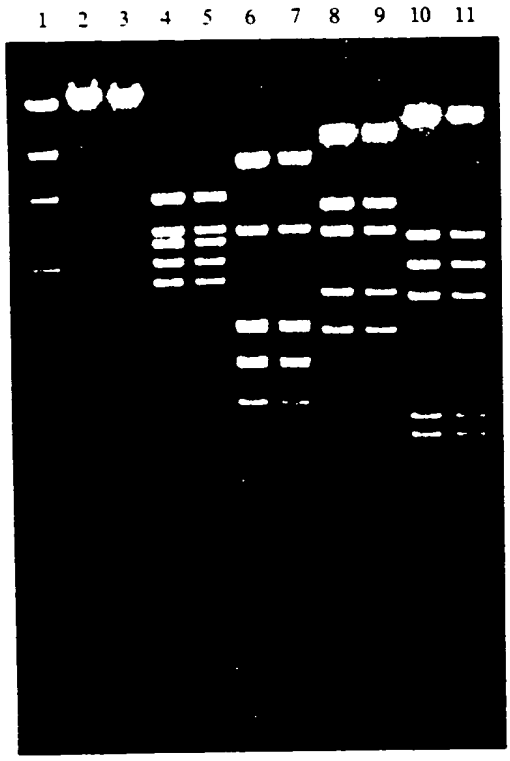

FIG. 3. Restriction pattern of cNeu-103 and cNeu-p clones. The cNeu-103 and cNeu-p clones were digested with *Eco*RI, and the 33-kb *Eco*RI inserts were gel-purified. The 33-kb *Eco*RI inserts were run on the agarose gel (lanes 2 and 3) or digested with *Bam*HI (lanes 4 and 5), *Bgl* I (lanes 6 and 7), *Hin*dIII (lanes 8 and 9), and *Xho* I (lanes 10 and 11) and then fractionated by gel electrophoresis. Lanes 2, 4, 6, 8, and 10, cNeu-103; lanes 3, 5, 7, 9, and 11, cNeu-p. λ *Hin*dIII markers are shown in lane 1.

Transfection of the DHFR clone followed by application of MTX allows selection of cells that have acquired a high copy number of the DHFR gene and of the cotransfected DNA (19). Six of 12 MTX-resistant colonies that were picked contained amplified *neu* sequences and high levels of p185. Fig. 2 shows the analysis of protein lysates from two cell lines derived from these colonies. These two cell lines exhibit the highest levels of gene amplification and antigen expression.

One of these two cell lines contains a 50- to 100-fold higher gene copy number than that of normal rat liver DNA and about 10-fold higher levels of p185 antigen than do colonies transformed by the cNeu-103 or cNeu-104 clone. Importantly, this colony was morphologically indistinguishable from untransfected NIH 3T3 cells (Fig. 1). The fact that very high level expression of the normal p185 antigen cannot alter the morphological phenotype of NIH 3T3 cells suggests that mutations leading to overexpression of the *neu* gene will not lead to oncogenic activation. We conclude that the alterations that activated the *neu* oncogene likely affect the structure of p185 rather than its regulation. They should eventually be found in the protein-encoding region of this gene.

DISCUSSION

We present here the molecular cloning of biologically active transforming and normal alleles of the *neu* gene. The fact that the entire *neu* gene resides in a single piece of 33-kb *Eco*RI fragment made it easy to construct a library that contains highly enriched *neu* gene. Using the 30- to 40-kb *Eco*RI fragments isolated from the primary mouse transfectant to construct the cosmid library, we found that ≈1 of 1000 colonies in the library contained the *neu* oncogene.

A high level of gene amplification of certain genes has been achieved by cotransfection with the DHFR gene followed by long-term growth in the presence of continually increasing concentrations of MTX (19). Such selection can be laborious and time-consuming. We describe here the success of gene amplification with a high copy number in a single step

selection. By cotransfecting the cNeu-p and pSV2-DHFR* clones and directly selecting the MTX-resistant colonies in relatively high concentrations (0.6 μM) of MTX, we have isolated the DHFR/G-8 cell line that contains ≈50–100 copies of cNeu-p and expresses a high level of p185 protein.

The DHFR/G-8 cells that contained amplified cNeu-p produced 10-fold higher levels of p185 than cells oncogenically transformed by cNeu-103 or cNeu-104. The fact that this very high level expression of normal p185 antigen does not morphologically alter the recipient NIH 3T3 cells suggests that the activation of the *neu* gene derives from a mutation in the protein-encoding region of the gene. The resulting altered p185 protein is able to induce transformation, even when it is expressed at relatively low levels in the cell.

The normal and oncogenic forms of the *neu* gene have no detectable differences in overall structure, as judged by extensive restriction endonuclease mapping. This shows that activation of the gene in the rat neuro/glioblastomas involved a very subtle change in DNA sequence. This idea is further supported by the data showing that the molecular weights of the p185 protein encoded by the normal and transforming alleles are very similar in size, as judged by $NaDodSO_4$ gel electrophoresis (Fig. 2).

One further clue concerning the nature of the activating lesion is provided by the fact that the carcinogen used to induce the rat neuro/glioblastomas was ethylnitrosourea, a known point mutagen (20). Taken together with the other data presented here, this suggests that subtle changes in p185 protein structure, like those found in the *ras*-encoded p21 proteins (21–28), may contribute to the outgrowth of certain types of tumors.

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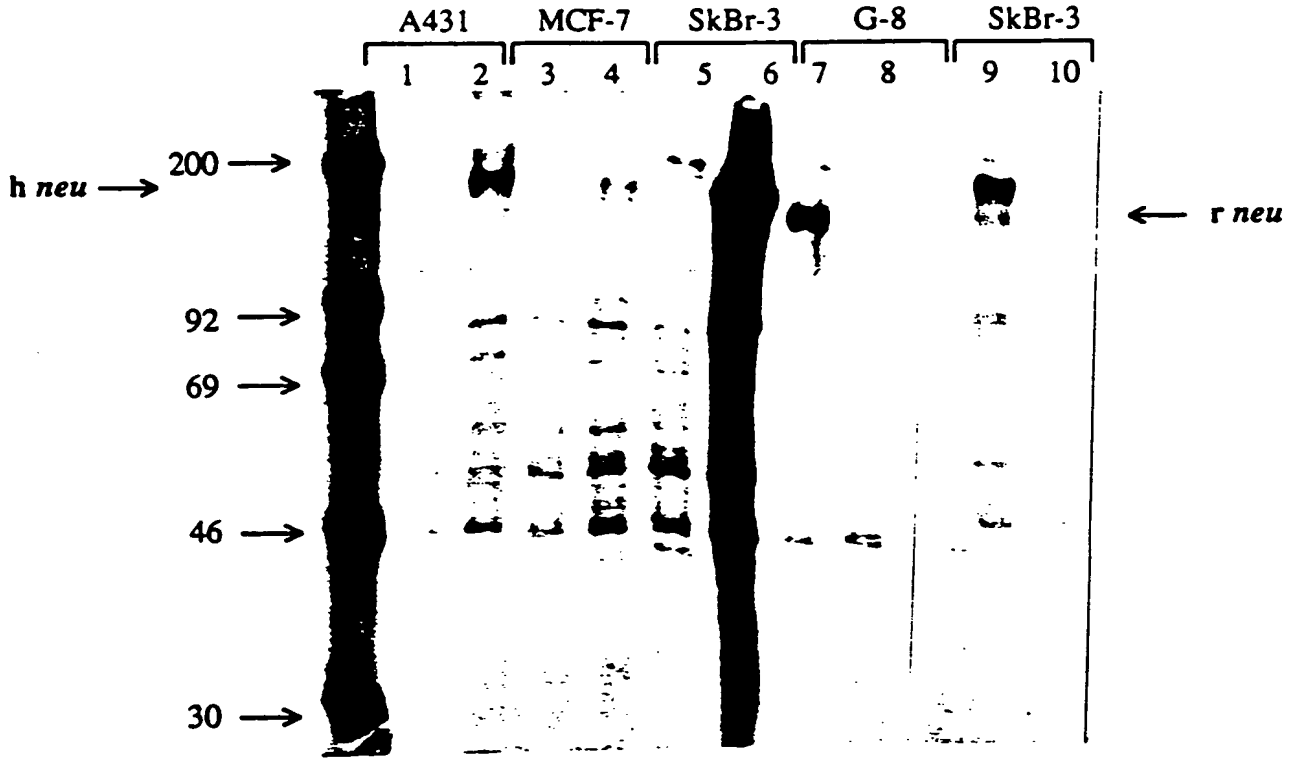
A431
MCF-7
SkBr-3
G-8
SkBr-3
1
2
3
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10
h neu
200
92
69
46
30
r neu

ATCC HTB 19 (continued)

Nude mouse: Forms grade II adenocarcinomas consistent with primary mammary cancer.
HLA cell line phenotype: A1; Bw16+/-.

REFERENCE SEED STOCK PREPARED AT ATCC

Number of Serial Subcultures from Tissue of Origin: 286.
Freeze Medium: Culture medium, 95%; DMSO, 5%; antibiotic-free.
Viability: 97%.
Culture Medium: Eagle's minimum essential medium with non-essential amino acids, sodium pyruvate and Earle's BSS, 90%; fetal bovine serum, 10%; antibiotic-free.
Isoenzymes: PGM_3, 1; PGM_1, 1; ES D, 1; AK1, 1-2; G6PD, B; GLO-1, 1-2.
Phenotype Frequency Product: 0.0115.
Sterility: Tests for mycoplasma, bacteria and fungi were negative.
Species: Confirmed as human by isoenzyme analysis.

ATCC HTB 20 BT-474 (Ductal carcinoma, breast, Human)

Current medium for propagation: RPMI 1640 with bovine insulin, 10 µg/ml, and glutamine, 300 mg/L, 90%; fetal bovine serum, 10%.

The BT-474 line was isolated by E. Lasfargues and W.G. Coutinho (J. Nat. Cancer Inst. *61:* 967-978, 1978) from a solid, invasive ductal carcinoma of the breast obtained from a 60 year-old female patient. The cells varied greatly in size and had large nuclei with one or more nucleoli. Microtubules, microvilli, tonofibrils, lysosomes, osmiophilic secretory granules, desmosomes, tight and gap junctions were observed in preparations examined by transmission electron microscopy. The BT-474 is reportedly tumorigenic in athymic nude mice and will form nodules in Amsterdam/IMR rats with regression in 10 days. This epithelial cell line was found to be susceptible to mouse mammary tumor virus (RIII-MuMTV) and can support its replication (In Vitro *15:* 723-729, 1979).

CHARACTERISTICS REPORTED FOR TRANSFERRED STOCK

Patient Data: Age-60; Sex-Female; Race-Caucasian.
Treatment: Surgery.
Grown as: Adherent patches of epithelial cells.
Morphology: Epithelial (compact, multi-layered colonies, rarely become confluent).
Karyology: Mode 55; range 50-112 (originator). Biomodal shift 58-59 and 100 in later passages with 3 marker chromosomes (Peterson).

REFERENCE SEED STOCK PREPARED AT ATCC

Number of Serial Subcultures from Tissue of Origin: 81.
Freeze Medium: Culture medium, 95%; DMSO, 5%.
Viability: 85-95%.
Culture Medium: RPMI 1640 medium, 90%; fetal bovine serum, 10%; bovine insulin, 10 µg/ml; glutamine, 300 mg/L.
Isoenzymes: G6PD, B; PGM_1, 1; PGM_3, 1; ES D, 1; Me-2, 0; AK1, 1; GLO-1, 1.
Phenotype Frequency Product: 0.0426.
Sterility: Tests for mycoplasma, bacteria and fungi were negative.
Species: Confirmed as human by isoenzyme analysis.

ATCC HTB 22 MCF7 (Breast adenocarcinoma, pleural effusion, Human)

Current medium for propagation: Eagle's MEM with non-essential amino acids, sodium pyruvate and Earle's BSS, 90%; fetal bovine serum, 10%.

Cells from the pleural effusion were seeded in Eagle's minimum essential medium with non-essential amino acids, 20 µg insulin per ml and 20% bovine calf serum for derivation of this line (H.D. Soule *et al.*, J. Nat. Cancer Inst. *51:* 1409-1416, 1973). It has retained several characteristics of differentiated mammary epithelium including ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes.

A culture at passage 138 was donated to the ATCC by C.M. McGrath in April, 1982. He has indicated that the line may harbor B- or C-type virus information and should be handled, therefore, as a potentially biohazardous agent.

REFERENCE SEED STOCK PREPARED AT ATCC

Patient Data: Age-69; Sex-Female; Race-Caucasian; Blood Type-O*.
Treatment: Radiation and hormone therapy.
Grown as: Monolayer; transferred 1:2 weekly.
Morphology: Epithelial-like.
Karyology: Chromosome Frequency Distribution 50 Cells: 2n = 46.

Cells:	1	1	2	2	4	2	3	1	3	5	7	4	6	5	2	2
Chromosomes:	66	70	72	73	75	76	77	78	80	81	82	83	84	85	86	87

The stemline chromosome numbers ranged from hypertriploidy to hypotetraploidy, with the 2S component occurring at 1%. There were 29-34 marker chromosomes per S metaphase, of which 24-28 markers occurred in at least 30% of cells, and generally one large submetacentric (M_1) and 3 large subtelocentric (M_2, M_3, and M_4) markers were recognizable in over 80% of metaphases. No DM's were detected. Chromosome No. 20 was nullisomic and X was disomic.
Isoenzymes: PGM_3, 1; PGM_1, 1-2; ES D, 1-2; AK1, 1; GLO-1, 1-2; G6PD, B.